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USE OF THE COMET ASSAY TO DETECT HYPOXIC CELLS IN MURINE TUMOURS AND NORMAL TISSUES EXPOSED TO BIOREDUCTIVE DRUGS

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The alkaline comet assay was applied to individual cells from mice exposed to two bioreductive drugs, tirapazamine and RSU 1069, with the goal of comparing DNA damage to tumours and normal tissues. More DNA single-strand breaks (SSBs) and a greater heterogeneity in DNA damage were observed in tumour cells than in spleen and marrow cells of mice exposed to 10–100 mg/kg tirapazamine, consistent with the presence of hypoxic cells and the greater bioreductive capacity of tumours. In mice injected with 25–200 mg/kg RSU 1069, aerobic cells exhibited large numbers of SSBs while toxic DNA interstrand crosslinks were produced only in hypoxic cells. Cells from bone marrow and spleen showed extensive numbers of SSBs, but minimal crosslinking compared to tumours where 10-20% of cells were heavily crosslinked. DNA damage produced by these two bioreductive drugs may be useful in estimating the range of individual cell oxygen contents within tumours and normal tissues.

Cells analyzed for DNA damage immediately after exposure to ionizing radiation contain SSBs related to their oxygenation status at the time of irradiation; aerobic cells show at least 3 times more strand breaks than radiobiologically hypoxic cells. The alkaline comet assay, a modification of a microgel electrophoresis method originally described by Östling & Johansson (1), has been used to detect individual hypoxic cells in murine and human tumours and normal tissues (2–5). However, the necessity of obtaining a tumour biopsy as rapidly as possible following a dose of radiation > 3 Gy prevents application of this technique for routine detection of hypoxic cells in human tumours.

In comparison to ionizing radiation, several bioreductive drugs show very large aerobic/hypoxic toxicity ratios; tirapazamine kills anoxic cells at concentrations up to 150 times lower than those required to kill the same fraction of well-oxygenated cells (6), and the hypoxic/oxic differential in cell killing of RSU 1069 can be 100 or more (7, 8). Both drugs are preferentially metabolized under anoxia by a variety of nitroreductases (9, 10), and reactive intermediates cause selective cell killing of hypoxic cells (7, 11). The selective DNA damage produced in hypoxic cells by tirapazamine (12, 13) and by RSU 1069 (14, 15) suggested that these drugs might prove effective in identifying hypoxic cells in solid tumours and normal tissues when applied in combination with a method which can measure DNA damage in individual cells. In fact, recent results using the comet assay validate this method for measuring the oxygenation of SCCVII murine tumours (16, 17). Since tirapazamine and a pro-drug of RSU 1069 (PD 144872) are being considered as adjuncts to radiotherapy in the clinic, it may be possible to use this approach to examine human tumour oxygenation.

In addition to analysis of DNA damage in tumour cells, the comet assay can be used to quantify DNA damage in normal tissues, therefore providing some measure of 'therapeutic ratio'. Bone marrow is believed to be the dose-limiting normal tissue (18) for tirapazamine, while gut toxicity has prevented the application of RSU 1069 and has led to development of less emetic analogues (19). Therefore measurement of damage to cells of bone marrow and jejunum in relation to other tissues should be informative. Experi-

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ments described below compare tirapazamine and RSU 1069 DNA damage to tumours and normal tissues.

Material and Methods

Tirapazamine (SR-4233; 3-amino-1,2,4-benzotriazine-1,4-dioxide) was kindly provided by Dr. Martin Brown, Stanford, and was dissolved in phosphate-buffered saline (PBS) at a concentration of 1 mg/ml (5.56 mM). RSU 1069 (1(2-nitro-1-imidazolyl)-3-aziridino-2-propanol) was originally supplied by Drs. T. Jenkins, I. Stratford and G. Adams of the MRC Radiology Unit at Harwell. Drug was freshly prepared at 5 mg/ml in PBS.

SCCVII squamous cell carcinoma cells were transplanted subcutaneously over the sacral region of inbred male C3H/HeN mice, approximately 30 g in weight and tumours were used at a size of approx. 500 mg. Mice were injected intraperitoneally with drugs and at various times, were sacrificed, tissues rapidly excised, and placed in ice-cold phosphate buffer. A single cell suspension was prepared from the entire tumour by mincing the tissue and incubating for 30 min with a mixture of trypsin, collagenase and DNase, and filtering the suspension through 50 μm nylon mesh (20). Spleen was removed and washed in ice-cold phosphate-buffered saline prior to mincing in ice-cold PBS containing 2 mM ethylenediaminetetraacetic acid (EDTA) and 2% dimethylsulphoxide. Bone marrow cells were removed from one tibia by purging with ice-cold saline using a syringe. Cells were filtered through 50 μm nylon mesh, centrifuged and resuspended in phosphate-buffered saline. For experiments examining DNA damage in tissues of mice asphyxiated after tirapazamine injection, mice were maintained for the 1-h treatment at 37°C. Cells were diluted to a concentration of about 2×10^4 cells/ml for the comet assay.

The alkaline comet assay was performed as previously described (4). Following agarose gel electrophoresis, individual cells or 'comets' were viewed using a Zeiss epi-fluorescence microscope attached to an intensified solid state CCD camera and image analysis system (2). The 'tail moment', defined as the product of the percentage of DNA in the tail multiplied by the tail length, and 'DNA content', defined as the total fluorescence associated with an image, were the most informative features.

Results

DNA strand breakage was produced by tirapazamine resulting in an increase in tail moment in individual cells from SCCVII murine tumour, bone marrow and spleen (Fig. 1a). Two to three times more SSBs were observed in tumour cells compared to spleen and bone marrow cells. With time after injection, SSBs were rejoined with similar kinetics in the three tissues (Fig. 1b). Comet histograms (for equivalent average damage levels) indicated that heterogeneity in DNA damage was somewhat greater in tumour cells compared to normal tissues but was smaller than expected for tumours containing both aerobic and hypoxic cells (Fig. 1c–e).

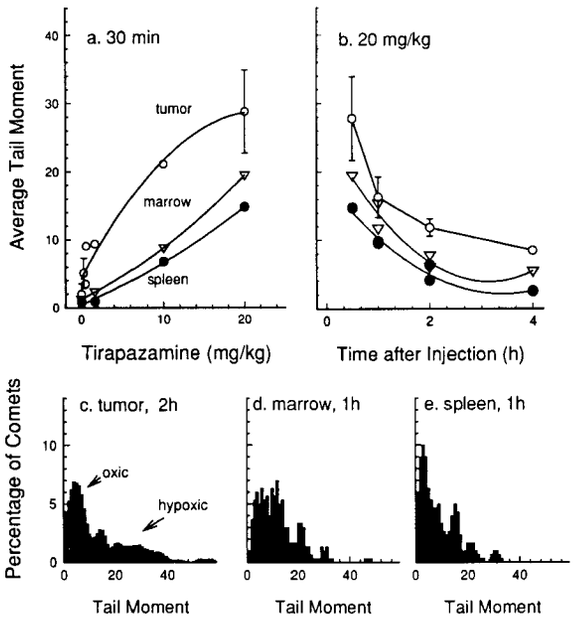

Fig. 1. Induction and rejoining of SSBs induced in SCCVII tumour and normal cells by tirapazamine. Panel a: Mice were injected i.p. with tirapazamine, and tumours and tissues were removed 30 min later for analysis of SSBs in individual cells using the alkaline comet assay. Average tail moment is proportional to SSBs, and a tail moment of 30 is observed following exposure to about 15 Gy . The mean and standard deviation for 3 independent experiments are shown for selected points. The remaining points show individual experiments in which 200 comets were analyzed. Panel b: tissues of mice exposed to 20 mg/kg tirapazamine were removed at subsequent times for analysis of SSBs. Panels c–e: histograms for cells from tumours and tissues exposed to 20 mg/kg and removed at the times indicated.

The greater sensitivity of tumours versus normal tissues to tirapazamine could be a result of differences in tissue oxygenation and/or bioreductase activity. To attempt to control for differences in tumour and normal tissue oxygenation, mice were asphyxiated 15 min following tirapazamine injection, and allowed to metabolize trapped drug for 1 h at 37°C. This experiment will provide an indication not only of reductase activity but also the amount of drug present in these tissues at the time of sacrifice. The amount of DNA damage observed 1 h after asphyxiation in normal tissues was significantly lower than the amount observed in tumours (Fig. 2), confirming differences in bioreductase activity. Interestingly, bone marrow cells showed fewer SSBs than spleen cells, in spite of greater damage observed in the marrow of air-breathing mice (Fig. 1a,b).

RSU 1069, unlike tirapazamine, produced significantly more SSBs in normal tissues than in tumours (Fig. 3).

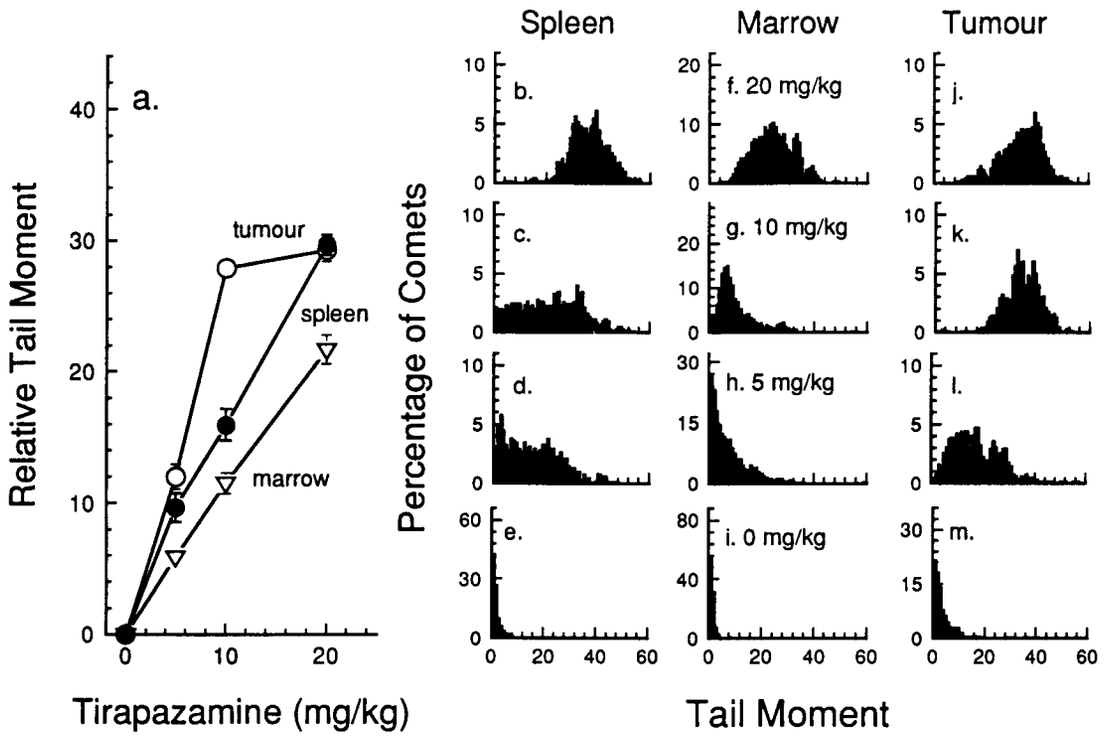

Fig. 2. Induction of damage in mice asphyxiated following administration of tirapazamine. Mice injected i.p. with tirapazamine were asphyxiated 15 min later, and then held at 37°C for 1 h prior to removal of tissues for analysis of SSBs. The mean (standard error) are shown for 3 independent experiments. Panels b-m show representative histograms for different tissues exposed to different concentrations of tirapazamine for 60 min after asphyxiation.

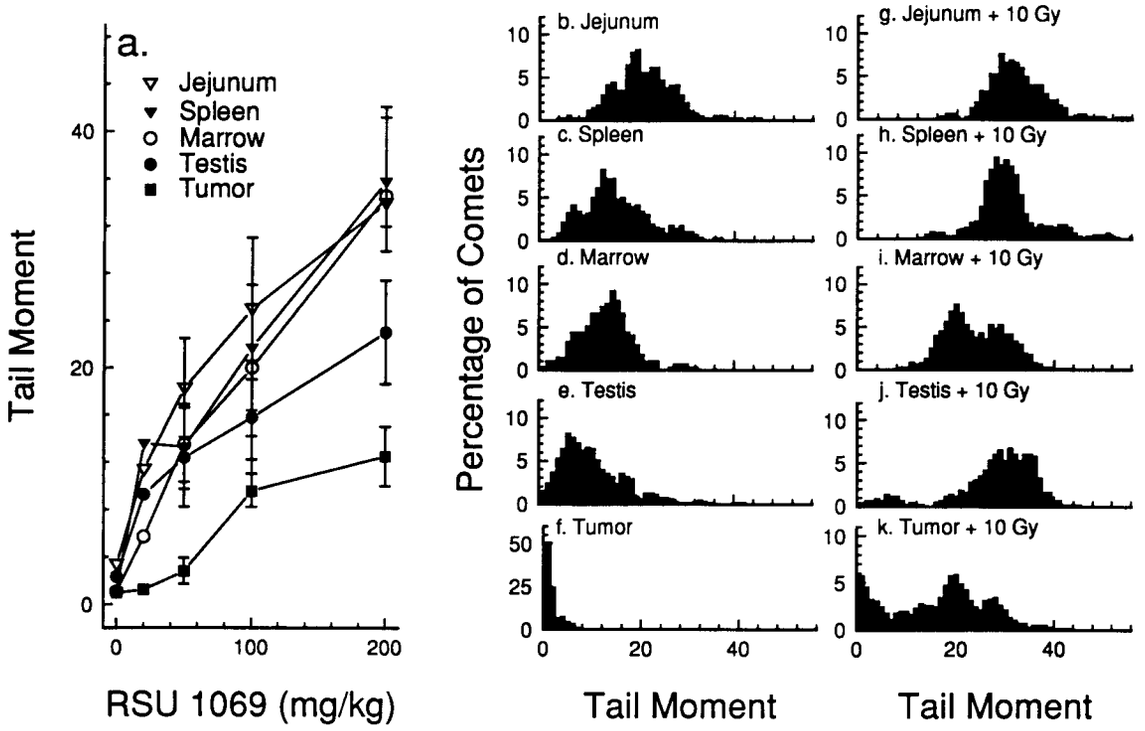

Fig. 3. Induction of DNA damage by RSU 1069 in SCCVII tumours and normal tissues. Mice were injected with RSU 1069 and 90 min later, tissues were recovered and single cells analyzed for DNA damage using the alkaline comet assay. Panel a: The mean and standard deviation for 3 independent experiments are shown for selected points. Panels b–f: representative histograms for single cells prepared from tissues of mice exposed to 50 mg/kg, and removed 90 min after drug injection. Panels g–k: histograms showing the response of the same population of drug treated cells exposed to 10 Gy in vitro.

Negligible DNA damage was observed in tumour cells of mice injected with doses less than 50 mg/kg. However, for tumour cells excised from these mice and exposed to 10 Gy in vitro, DNA in a proportion of the tumour cells still failed to migrate indicating the presence of DNA interstrand crosslinks (Fig. 3k). Testis cells showed significantly less DNA damage than cells of other normal tissues, and like tumour cells, there was evidence of a small percentage of undamaged cells after irradiation in vitro (Fig. 3j). Cells from jejunum, spleen and marrow showed no evidence of crosslinks (Fig. 3g–i).

Discussion

As might be expected on the basis of relative bioreductive capacity and presence of hypoxic cells, SCCVII tumour cells showed more DNA damage by tirapazamine than bone marrow or spleen cells. However, much less heterogeneity in damage was observed in tumour cells than expected on the basis of an oxic/hypoxic differential of 40 which was previously observed for spheroids exposed to tirapazamine and examined for DNA damage using the comet assay (16). Possible explanations for the reduced heterogeneity include hypoxia induced during excision when tirapazamine is still present, a relatively small differential in tumour oxygenation (perhaps 0.1% to 5%), transient changes in tumour oxygenation (acute hypoxia) during drug metabolism, or decreased drug accessibility of hypoxic cells distant from the blood supply.

The 'therapeutic ratio', in terms of DNA damage, favours damage to SCCVII tumour cells by a factor of 2 or more, although the exact relation between DNA damage measured by the comet assay and cell killing by tirapazamine has yet to be defined for normal tissues. More damage was induced in bone marrow than spleen cells of air-breathing mice (Fig. 1). The opposite relation was observed in asphyxiated animals suggesting that there may be a population of less well-oxygenated cells in bone marrow. Bone marrow suppression is likely to be the dose limiting toxicity for tirapazamine (19), and there are indications from other studies that some bone marrow cells may border on hypoxia (21). In mice breathing 10% oxygen, radiation-resistant hypoxic cells were observed in the bone marrow but not spleen using the comet assay (4).

For RSU 1069, normal tissues consistently showed more SSBs than tumours, but only testis and tumour exhibited DNA crosslinks, the presumed cytotoxic lesion (7, 14). While emesis is dose-limiting in patients, the cell mixture obtained from the jejunum did not exhibit more damage than other normal tissues. Damage by the alkylating group of RSU 1069 which is responsible for SSBs is unlikely to be dependent on oxygen or nitroreductase level so that the decreased DNA damage in aerobic tumour cells relative to normal tissues (Fig. 3f) would seem to indicate less access of tumour cells to RSU 1069. Assuming that single-strand breaks should occur as frequently in aerobic tumour cells as in normal tissues, results shown in Fig. 3a suggest that tumour exposure to RSU 1069 is reduced by at least a factor of 2 compared to well-vascularized normal tissues. The reduced sensitivity of the testis cells may be a result of hypoxia, decreased drug accessibility, and/or decreased ability of the drug to alkylate highly compacted DNA. The highly condensed state of chromatin and exchange of histones with protamines which are more efficient radical scavengers are often used to explain the resistance of testis cells to DNA damage by ionizing radiation (22). Presumably these differences might also affect sensitivity of testis cells to RSU 1069.

A comparison between damage produced by the two drugs in spleen and bone marrow is informative. RSU 1069 appears to produce a similar amount of DNA damage in spleen as in bone marrow cells, with no evidence of extensive crosslinking indicative of hypoxia. Tirapazamine, on the other hand, appears sensitive to a difference between these two tissues, with bone marrow showing indirect evidence of the presence of hypoxic cells. The greater apparent sensitivity of tirapazamine for detecting borderline hypoxia can be explained by the difference in oxygen sensitivity of metabolic activation of tirapazamine and RSU 1069. Tirapazamine continues to undergo some reduction even at high oxygen concentrations, but RSU 1069 crosslinking is confined to cells with relatively low oxygen contents (23). Thus, for detecting cells bordering hypoxia, tirapazamine, like ionizing radiation, should be more effective than RSU 1069. However, RSU 1069 appears more able than tirapazamine to resolve a distinct 'hypoxic fraction' in tumour cells. For tirapazamine, the resolution between aerobic and hypoxic cells is indistinct and is dependent on both drug dose and time after injection. However, for RSU 1069, hypoxic tumour cells are clearly those with crosslinks detectable after exposure to 10 Gy. The hypoxic fraction determined in SCCVII tumours using RSU 1069 crosslinking is in good agreement with the hypoxic fraction measured using radiation to induce damage, or using the standard paired-survival curve method (17). While the amount of crosslinking caused by RSU 1069 will be dependent not only on presence of hypoxic cells but also on nitroreductase activity, the proportion of cells containing crosslinks should be relatively independent of bioreductive capacity.

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